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Postmating reproductive barriers contribute to the incipient sexual isolation of US and Caribbean *Drosophila melanogaster*

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2 ***Drosophila melanogaster***

3

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11   *Abstract*

12   The nascent stages of speciation start with the emergence of sexual isolation. Understanding

13   how reproductive barriers influence this evolutionary process is an ongoing effort. We present

14   here a study of *Drosophila melanogaster* populations from the southeast United States and

15   Caribbean islands undergoing incipient sexual isolation. The existence of premating

16   reproductive barriers have been previously established, but they do not fully account for the

17   degree of isolation present. To assess the influence of postmating barriers, we investigated

18   putative postmating barriers of female remating and egg laying behavior, as well as hatchability

19   of eggs laid and female longevity after mating. While we did not find any effects in female

20   remating or egg laying, we did observe lower hatchability in the central region of our

21   geographical spread as well as shorten female life spans after mating to genetically different

22   males in females originating from the northern- and southernmost locations of those surveyed.

23   These results serve as evidence that long-term consequences after mating such as the fitness

24   of offspring and shortened lifespan have a stronger effect than short-term postmating behaviors.

25

26   *Running Title:*

27   **Postmating barriers in US and Caribbean fruit flies**

28

29   *Key Words*

30   **sexual conflict, egg laying, hatchability, remating, sperm toxicity, chase away selection**

31

32 *Introduction*

33 Males and females in sexual mating systems ultimately have different reproductive interests and this

34 interaction of contrasting interests is commonly referred to as sexual conflict (Chapman et al. 2003;

35 Parker 1979). Competitive males are selected to override the mating preferences evolved by females

36 (Holland and Rice 1998). Females consequently evolve resistance to male 'coercion' tactics, and males

37 are then selected by novel or more exaggerated traits - perpetuating an endless evolutionary chase

38 between the sexes (Arbuthnott et al. *2014*; Parker 1979; Civetta and Singh 1995; Rice 1996; Chapman et

39 al. 2003; Arnqvist and Rowe 2005). This phenomenon of conflict in reproductive optima has been

40 experimentally demonstrated to promote an antagonistic male-female coevolution that is the essence of

41 sexual isolation which precedes speciation (Chapman et al. 2003; Parker 1979; Holland and Rice 1998).

42

43 The tempo and mode by which new species are formed are influenced by the development of

44 reproductive barriers, which facilitate reproductive isolation. When multiple barriers accumulate, gene flow

45 is reduced between or within a given population, which can give rise to early stages of speciation. These

46 reproductive barriers are traditionally classified as those occurring before fertilization, prezygotic, and

47 those occurring after fertilization, postzygotic (Coyne and Orr 2004). The latter can be further broken

48 down into extrinsic and intrinsic depending on if the barrier interacts with external factors (i.e.

49 environmental, individuals) or internal factors (i.e. genetic incompatibilities) (Seehausen et al., 2014).

50 Speciation involves multiple reproductive barriers of varying effect sizes (Coyne and Orr 2004,

51 Seehausen et al., 2014), and identifying the interactivity and varying strengths of reproductive barriers at

52 play is vital to characterizing the process of speciation.

53

54 Flies from the *Drosophila* genus are particularly suited to study the rise and fall of reproductive barriers

55 since the range of study systems span the whole speciation spectrum from completely non-interbreeding

56 species to hybridizing species (Bono and Markow, 2009), as well as species experiencing sexual isolation

57 within itself (Yukilevich and True, 2008b). Empirical studies investigating sexual selection in *D.*

58 *melanogaster* have been conducted about the evolution of prezygotic isolation - mate choice, male

59 morphology, and courtship behavior (Yukilevich and True 2008a; Hollocher 1997). Is has been suggested

60   that premating barriers are of greater strength (Cozzolino and Scopece 2008; Coyne and Orr 1989;

61   Ramsey *et al.* 2003; Coyne and Orr 2004). Postzygotic barrier mechanisms have also been known to

62   have an influence in several systems of *Drosophila* flies, but has mostly been studied in hybridizing

63   species such as *D. mojavensis/D. arizonae* (Bono and Markow, 2009) and *D. melanogaster/D. simulans*

64   (Matute et al., 2014). The emergence of postzygotic barriers within a single species has been somewhat

65   less investigated. However, the current knowledge of postmating consequences in *Drosophila*

66   *melanogaster* suggests that this species is a good model to investigate emerging postmating reproductive

67   barriers.

68

69   Male sperm transferred after matting contain accessory gland proteins reduce female remating rates and

70   increase egg laying (Chapman et al. 2003; Wolfner, 1997). Reduced receptivity to remating will also

71   decrease the female opportunity to mate with another male that may result in more fit progeny. Increased

72   egg laying and the trauma from mating reduces female lifespan (Fowler and Partridge 1989). It is even

73   suggested that male sperm is toxic to females (Rice 1996). As a result, females develop resistance to

74   these harmful male traits, and males subsequently evolve new methods to discourage females from

75   mating with other males (Arnqvist and Rowe 2005). It has been suggested that females should be more

76   resistant to males they have co-evolved in with compared to males they have not coevolved with. These

77   effects vary across populations, and ecological context appears to be a factor (Arbuthnott et al. 2014).

78   This rapid, cyclical process termed sexually antagonistic coevolution has been demonstrated in not only

79   Drosophila species (Knowles and Markow 2001) and water striders (Rowe et al. 2002), but also in many

80   other animals. Coevolution by sexual conflict is a strong force behind reproductive isolation, which may

81   lead to speciation in specific circumstances (Martin and Hosken 2003).

82

83   Studying areas of where secondary contact where once genetically isolated, allopatric populations

84   interbreed is a powerful approach to understanding the strength and dynamics of male-female postzygotic

85   divergence (Jiggens and Mallet 2000). Of particular interest to this subject are the Caribbean islands and

86   southeastern United States. This area is a secondary contact zone of west African and European *D.*

87   *melanogaster* populations, which have evolved in allopatry for ~10,000-15,000 years (Capy et al. 1986).

88   Secondary contact occurred in two waves with west African flies migrating with the transatlantic slave

89   trade 400-500 years ago to the Caribbean islands and the cosmopolitan flies arriving to the east coast US

90   with European colonists <200 years ago (Capy et al., 1986; Duchen et al., 2013). It is known that in this

91   species members of the African Zimbabwe population very rarely mate with individuals outside of this

92   population, which are commonly referred to as 'cosmopolitan' (Hollocher et al. 1997). The contrasting

93   natures of African and cosmopolitan flies are reflected in the Caribbean and southeast US flies

94   respectively with partial mating isolation between west African flies and US cosmopolitan flies (Yukilevich

95   and True 2008a). Previous authors have noted peculiar morphological, behavioral, and pheromonal

96   differences in these North American populations where Caribbean populations display exceptional

97   African-like morphology unlike those from the United States, which have retained cosmopolitan

98   phenotypes from their European predecessors (Yukilevich and True 2008a; Capy et al. 1993; David and

99   Capy 1988). Recent microsatellite evidence also indicates that United States flies are more genetically

100   similar to African flies than are European flies, suggesting that African alleles may have introgressed into

101   North America via the Caribbean islands (Duchen et al., 2013; Yukilevich and True 2008b; Caracristi and

102   Schlotterer 2003; Capy *et al.* 1986) supporting the existence of a southeast US and Caribbean island

103   'hybrid' zone between African and European populations. Only mating preferences and other

104   premating/prezygotic reproductive barriers have been formally treated in this system (Yukilevich and True

105   2008a; Yukilevich and True 2008b), and the presence of incipient postmating sexual isolation in

106   Caribbean populations remain unexplored.

107

108   We present our study as an effort to better understand the role of postmating reproductive barriers in a

109   system of *Drosophila melanogaster* experiencing incipient sexual isolation. We have investigated the role

110   of remating, female egg laying, hatchability of laid eggs, and female longevity after mating with different

111   males as putative postmating reproductive barriers. These phenotypes are good candidates to investigate

112   the scope of extrinsic and intrinsic postmating reproductive barriers. We measure each of these

113   phenotypes in females from different locations in the southeast US and Caribbean islands and examine

114   them for geographical patterns, which may reveal if and how these barriers affect this system of

115   southeast United States and Caribbean Island *Drosophila melanogaster*.

116

117 **Materials and Methodology**

118 *Fly Lines and Rearing Conditions*

119 For our phenotypic assays, we used 23 isofemale lines of *Drosophila melanogaster* collected in the

120 summer of 2004 and 2005 (Yukilevich and True 2008). Origins are as following (TABLE 1; FIGURE 1):

121 Birmingham, AL (lines 1-1 and 1-2); Selba, AL (lines 2-1 and 2-2); Thomasville, GA (lines 3-1 and 3-2);

122 Meridian, MS (lines 4-1 and 4-2); Tampa Bay, FL (lines 5-1 and 5-2); Sebastian, FL (line 6-1); Freeport,

123 Grand Bahamas-west (lines 7-1 and 7-2); Bullock's Harbor, Berry Islands (lines 8-1 and 8-2); Cockburn

124 Town, San Salvador (lines 9-1 and 9-2); George Town, Exumas (lines 10-1 and 10-2); Mayaguana,

125 Mayaguana (lines 11-1 and 11-2); Port Au Prince, Haiti (lines 12-1 and 12-2). Original line ID numbers

126 are listed in Supplemental Table 1. All flies were maintained at 25 °C in vials on a standard cornmeal diet

127 (recipe available upon request) and entrained under a 12hr light:12hr dark regime.

128

Map Number	Location	Line(s)
1	Birmingham, AL	1-1 and 1-2
2	Selba, AL	2-1 and 2-2
3	Thomasville, GA	3-1 and 3-2
4	Meridian, MS	4-1 and 4-2
5	Tampa Bay, FL	5-1 and 5-2
6	Sebastian, FL	6-1
7	Freeport, Grand Bahamas - West	7-1 and 7-2
8	Bullock's Harbor, Berry Islands	8-1 and 8-2
9	Cockburn Town, San Salvador	9-1 and 9-2
10	George Town, Exumas	10-1 and 10-2
11	Mayaguana, Mayaguana	11-1 and 11-2
12	Port Au Prince, Haiti	12-1 and 12-2

129 Table 1: Corresponding map locations and lines

130

6

131   *Egg laying, Hatchability, and Remating Rate Assays*

132   Virgin females were collected from all 23 isofemale lines. Male flies up to one day old were collected from

133   two lines (lines 1-2 and 11-1) located at polar ends of our geographical study region. We chose these two

134   lines as sources for male flies based on clinal distance as well as maximal differences between courtship

135   profiles and physical characteristics (Yukilevich and True 2008b) to account for female mate preference

136   which has been previously established (Yukilevich and True 2008a). All flies were collected on light CO2

137   anesthesia and aged for three to four days before entering our assays. We set up a full factorial

138   experiment where females from each of the isofemale lines were crossed with the two lines from which

139   males were collected. Each cross was replicated 15 times.

140

141   All flies were live manipulated using aspirators for the remainder of the phenotypic assays to avoid any

142   physiological and behavioral effects of CO_2 anesthesia (Badre *et al.* 2005). Assays lasted 24 days and

143   were conducted in two stages. The first stage measured female remating rates and egg laying rates over

144   a 10-day period; during the following 14 days, second stage quantified hatchability rates. In the first stage

145   (i.e. first 10 days), females were transferred daily by aspirator into new vials with standard cornmeal fly

146   food and blue food coloring. The dye helped visualize eggs laid by females without causing any variability

147   in their behavior (Bergland 2012). The vials also had 20 uL of a 10% diluted active yeast mixture to

148   stimulate females' reproductive activity.   At lights on (i.e. dawn) on the initial day of the first stage,

149   individual females were aspirated into a vial with two males from either one of the two selected male lines

150   for mating. Approximately 90 minutes were allocated for copulation to occur, and all males were

151   discarded immediately after this time period using an aspirator. Females that did not mate on the first day

152   did not continue in the assay. Fecundity assays were conducted daily after the females were transferred

153   into new vials. To assess short-term and long-term receptivity to remating effects, each individual female

154   was introduced to two new males of the same genotype from her initial mating on the fourth and eighth

155   day of the assay (i.e. three and seven days after initial mating). We allowed 90 minutes on both re-mating

156   days for copulations to occur and all males were discarded via aspirator thereafter.

157

158   On the first day of the second stage of the assay in order to ensure the quality of our phenotypic dataset,

7

159   female identities were checked to confirm correct sexing from when males were discarded from remating

160   days. Incorrectly sexed vials in which the female - instead of the male - were accidentally discarded were

161   not included in later analysis. Remaining vials that passed the first stage of the experiment were

162   monitored daily for fly eclosion. Flies that eclosed were recorded and discarded immediately. Fly eclosion

163   monitoring was terminated when either three consecutive days of zero fly eclosions or 14 days of

164   monitoring was reached - whichever came first. All phenotyping assays during the first and second stages

165   were conducted within the first three hours of lights on (i.e dawn). All flies from the first stage and eclosing

166   vials in the second stage were incubated at a controlled 25 °C with a light timer set for a 12hr light: 12hr

167   dark cycle.

168

169   *Longevity Assays*

170   For our longevity assays, we phenotyped a subset of lines from the 23 isofemale lines that spanned the

171   southeast United States and Caribbean Islands. Female flies used in our longevity assays originated from

172   (arranged from north to south) Selba, Alabama, USA (line 2-2), Thomasville, Georgia, USA (line 3-1),

173   Freeport, Grand Bahamas-west (line 7-2), Bullock's Harbor, Berry Islands (line 8-1), and Port Au Prince,

174   Haiti (line 12-2). Representative 'American' and 'Caribbean' males were derived lines originating from the

175   same male collection lines used in egg laying, hatchability, and remating assays, i.e. Birmingham,

176   Alabama, USA (line 1-2) and Mayaguana, Mayaguana (line 11-1), respectively. 'Homotypic' crosses were

177   defined as male and female both of either American or Caribbean origin. "Heterotypic" crosses were

178   defined as male and female from different origins (i.e. American x Caribbean or Caribbean x American).

179   Male and females from the same origin were assumed to be more related and genetically similar to each

180   other than those from different origins based on previous evidence (Yukilevich and True 2008b).

181

182   Virgins females were collected on light CO_2 anesthesia and aged singly in vials for four days. Males were

183   collected in the same manner and aged in groups of five per vial. We performed crosses in two separate

184   rounds, which lasted approximately 70 and 80 days, respectively. In the first round, we crossed female

185   flies from Selba, Alabama, USA and Port Au Prince, Haiti to either our representative 'American' male or

186   'Caribbean' male. There were 50 replicates for each unique cross. Because of the large effect size from

187 our initial round, in the rest of our lines we had 25 replicates for each type of cross. In each round, aged

188 female flies were placed with five male flies for 48 hours to ensure mating occurred. Male flies were

189 discarded using an aspirator after the mating period. Female flies were then observed on a regular basis

190 five days per week. Dates of deaths were recorded until the end of the 70 or 80-day observational period.

191 The females were transferred to fresh vials every seven days.

192

193 *Post-mating behavior data analysis*

194 We examined the effects of geographic location on the total number of eggs laid by females, the total

195 hatchability of those egg laid, and the propensity of females to remate three and seven days after initial

196 mating. For egg laying and hatchability, we used a linear regression model with latitude and longitudinal

197 coordinates as predictors as well as the male and female identity and phenotyping blocks to account for

198 the variation from genotypes of male and females in addition to experimental block effects. Model fit and

199 effects of factors was assessed with ANOVA tables produced by the models. Because remating was

200 scored as a categorical variable of whether or not the female copulated on the two remating days, we

201 used logistic regression models to assess the effects of geographic location while controlling for male and

202 female genotypes and block effects on short- and long-term female receptivity to remating. The

203 significance of longitudinal and latitudinal coordinates and model fits were assessed using analysis of

204 deviance tables.

205

206 We performed a permutation test to investigate the significance of the lower hatchability rates in the three

207 central locations as revealed by linear models as well as visual confirmation of plots. We calculated the

208 difference in hatchability between the five lines from our three central locations and the hatchability of all

209 other fly lines (18 lines). We then randomly assigned fly lines into groups of five and 18 and calculated the

210 difference in hatchability between these two groups. These permutations were repeated 10,000 times. P-

211 values were calculated by the number of times the difference in hatchability between these two groups

212 were equal to or greater than our observed value divided by our 10,000 permutations. The line with the

213 lowest hatchability was removed for a follow-up permutation test to confirm that the lower hatchability was

214 only due to the effect of one line. Similar permutation tests was conducted on total egg counts to

9

215   determine that lower hatchability was also not due to lower egg counts. Hatchability of eggs laid by

216   females mated to representative 'American' and 'Caribbean' males were performed separately, and P-

217   values from these tests were corrected using the Bonferroni method.

218

219   All analysis was performed in R and the code for the permutation test is available upon request.

220

221   *Longevity data analysis*

222   Survival analysis is used for temporal data of waiting times to an event with censored data. We employed

223   methods from survival analysis to examine our data. We analyzed the waiting times of female death after

224   homotypic or heterotypic mating. Females that escaped or survived past our observational periods were

225   considered censored data points. The first step of survival analysis is to estimate survival functions for

226   each of our crosses, S(t), which in our study is the probability of a female living longer than time, t. This

227   can be done non-parametrically using the Kaplan-Meier method (Kleinbaum and Klein 2012). Parametric

228   models were tested (i.e. exponential, log-normal, log-logistic, and generalized gamma), but none yielded

229   a good fit (data not shown). After survival curves were fitted, we used it to estimate the cumulative hazard

230   function, H(t), for each type of cross. The cumulative hazard function shows the cumulative probability

231   that a female has expired up to time, t. The relationship between the survival function and the cumulative

232   hazard function is:

233

$$H(t) \ = \ -ln(S(t))$$

234

235   or

236

$$S(t) \ = \ e - H(t)$$

237

238   The most common statistical test used for comparing survival distributions is the log-rank test. However,

239   this test has the proportional hazards assumption which requires that the hazard functions of the two

240   groups being compared are parallel. Hazard functions for our comparisons of female longevity after

10

241   heterotypic and homotypic matings were plotted and visually checked for the crossing of hazard curves.

242   When hazard curves cross, the proportional hazards assumption is violated so another test must be

243   conducted because the standard log-rank test has little to no power (Klein and Moeschberger 1997). We

244   chose to use a combined weighted log-rank test, which takes into account crossing hazard curves (Zhou

245   et al., 2006). This improved log-rank test has more power than the standard log-rank tests when the

246   hazard functions cross and the hazard ratio is not proportional.

247

248   All analysis was performed in R using the 'survival' package to estimate the survival curves and hazard

249   functions. The package 'emplik' was used as part of the improved log-rank test where the R code can be

250   obtained online (http://www.ms.uky.edu/%7Emai/research/LogRank2006.pdf).

251

252   **Results**

253   *Egg counts*

254   Egg counts for each line are shown using side-by-side box plots with locations arranged from the

255   northernmost to the southernmost location, left to right (FIGURE 2A, 2B). It does not appear that egg

256   counts follow a clinal pattern in either case of females mated to representative 'American' or 'Caribbean'

257   males. There is much variation within lines, but the median egg count for each location is approximately

258   the same except for in the case of females from location 6 (line 6-1; Sebastian, FL) when mated to

259   Caribbean males (FIGURE 2B).

260

261   The full regression model showed that longitude and latitude were not significant influences (p = 0.3324)

262   on egg laying and that most variance was accounted for by male (p < 0.001) and female (p<0.001)

263   genotypes as well as block effects (p = 0.0018). Comparing the full model with the reduced model in

264   which longitude and latitude were omitted showed that the addition of longitude and latitude as predictive

265   variable did not help the predictive power of the full model (p = 0.4994). (SUPPLEMENTARY TABLE 2, 3,

266   4)

267

268   *Remating*

11

269   Short- and long-term remating rates for each isofemale line were plotted against latitude and longitude

270   coordinates (SUPPLEMENTARY FIGURE 1, 2). Short-term remating rates were generally lower (range of

271   rates : 0-30%) than long-term remating rates (range of rates: 0-60%). Remating rates do not appear to be

272   influenced by location, which was investigated further with logistic regression.

273

274   The full logistic regression model evaluating effects of latitude and longitude while controlling for male and

275   female genotypes as well as block effects found that latitude ($p = 0.11$) or longitude ($p = 0.35$) were not

276   useful in predicting short-term remating rates with similar results for long-term remating rates (lon $p =$

277   0.7616, lat $p = 0.6361$). Male genotype also was not a significant influence on short-term or long-term

278   remating rates ($p = 0.4848$ and $p = 0.1240$). The reduced models removing latitude and longitude as

279   predictors also showed that they were not significantly influencing remating rates. Female identities in

280   both logistic models for short- and long- term remating rates were significant giving evidence that female

281   genotypes could influence remating rates. However, when we fitted a model for long-term remating rates

282   with a male x female interaction term, results showed that this interaction term was not significant ($p =$

283   0.0959). (SUPPLEMENTAL TABLE 5, 6, 7, 8, 9, 10, 11, 12)

284

285   *Hatchability*

286   Hatchability for the various locations in the southeast US and Caribbean Islands were visualized using

287   side-by-side box plots with locations arranged from the northernmost to the southernmost location, left to

288   right (FIGURE 2C, 2D). Hatchability in the three middle locations (location 4, 28, 33) at the border of the

289   southeast US and Caribbean Islands appear lower than the locations on the edges in both the graphs

290   displaying hatchability of females mated to American males (Figure 2C) and Caribbean males (Figure

291   2D).

292   Our full linear regression model took into account male and female identities on hatchability as well as

293   experimental block effects while assessing influences of longitude ($p = 0.048$) and latitude. Longitude had

294   a significant effect on hatchability ($p =0.0483$) while latitude did not ($p = 0.2396$). However when we

295   compare the reduced model with latitude removed with the full model, we find that latitude did help

296   significantly in explaining hatchability (0.0302). (TABLE 2, 3, 4)

12

297

298

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Block	14	2.9337	0.20955	4.6414	4.007e-08
Female	22	7.7220	0.35100	7.7745	<2.2e-16
Male	1	0.8869	0.88692	19.6448	1.084e-05
Latitude	1	0.6255	0.06255	1.3856	0.23956
Longitude	1	0.17674	0.17674	3.9147	0.04826
Residuals	694	31.3326	0.04515		

299   TABLE 2: ANOVA Table for Full Model of Hatchability

300

301

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Block	14	2.9337	0.20955	4.6168	4.557e-08
Female	22	7.7220	0.35100	7.7332	<2.2e-16
Male	1	0.8869	0.88692	19.5403	1.143e-05
Longitude	1	0.0264	0.02639	0.5813	0.446
Residuals	6945	31.5455	0.04539		

302   TABLE 3: ANOVA Table for Reduced Model of Hatchability with no Latitude

303

	Res Df	RSS	DF	Sum of Sq	F	Pr(>F)
Full	694	31.333				

13

Reduced	695	31.546	-1	-0.21291	4.47158	0.03022

304 TABLE 4: ANOVA table of model comparisons

305

306 To evaluate the significance of the dip in hatchability rates, we performed permutation tests as described

307 in our methods section. We found that the hatchability in the middle three locations was significantly

308 lower than the rates in the surrounding locations regardless of being mated to an American male (p <

309 0.0001) or Caribbean male (p <0.0001). Results were similar when the location with the lowest

310 hatchability rate was removed (28: Sebastian, FL, USA) and the permutation tests performed again

311 (females mated to American male: p = 0.0056, females mated to Caribbean male: p = 0.0272). Similar

312 tests were conducted on egg counts to investigate whether the lower hatchability was due to lower egg

313 counts. No significant differences in egg counts between females from the middle locations and the outer

314 locations were found regardless of whether they were mated to American males (p = 0.3192) or

315 Caribbean males (p = 0.7584). The same results were yielded when we removed the influence of the

316 middle location, 28: Sebastian, FL, USA, (mated to American males: p=0.3016, mated to Caribbean

317 males: p = 1.0). These results suggest a generalizable central location effect on hatchability.

318

319 *Longevity*

320 Five female lines representing various locations in the Southeastern U.S. and Caribbean Islands were

321 measured for longevity after experiencing homotypic or heterotypic matings. The homotypic cross survival

322 curves for females from lines 3-1, 2-2, and 12-2 were consistently higher than the survival curves of

323 females in heterotypic crosses (FIGURE 3, 4, 5). There were no apparent differences between homotypic

324 and heterotypic survival curves of females originating from lines 7-2 or 8-1 (Figure 6, 7).

325

326 Hazard curves for all crosses and lines revealed non-proportional hazards in almost all cases of

327 homotypic and heterotypic matings. (SUPPLEMENTARY FIGURE 3, 4, 5, 6, 7). Crossing points of all

328 hazard functions were visually estimated for use in the improved log-rank tests (TABLE 5). The improved

329 log-rank tests showed evidence that females after heterotypic matings had shorter lifespans than females

330 in homotypic matings for females from lines 3-1 and 12-2 (p = 0.0410 and p = 0.0271). Females of line 2-

14

331 2 showed a reduced lifespan when involved in heterotypic matings (FIGURE 3), but these results were

332 not significant (p = 0.3130).

333

Female line	T~approx time of crossing hazards	pvalue from improved log rank
13,34	37	0.04096407
40,23	42	0.4246727
33,11	40	0.6260448
H,25	23	0.02706502
20,17	61	0.3129819

334 TABLE 2.5: Improved Log-rank Test Results

335

336 **Discussion**

337 Speciation is a complex process, dependent on a plethora of factors including diverse selectional forces

338 and interactions with the environment. We examined several potential postmating reproductive barriers

339 including remating rates, egg laying rates, hatchability, and female longevity that may potentially influence

340 a system in the early stages of sexual isolation.

341 We observed an interesting hatchability rate 'valley' produced by the isofemale lines originating from our

342 three central locations spanning the border of the United States and the Caribbean Island. This result

343 may be evidence that there are essential genetic differences between American and Caribbean fly

344 populations, which could have manifested as an intrinsic postzygotic barrier between these two

345 populations. This type of evidence is indicative of the presence of Bateson-Dobzhansky-Muller

346 incompatibilities (DMI) which are negative epistatic interactions and the most common form of intrinsic

347 postzygotic isolation (Presgraves, 2010). A reduction in the fitness of 'hybrid' offspring here restricts the

348 product of gene flow between American and Caribbean *D. melanogaster* populations. A more thorough

349 investigation of these lines and genome sequences that are beyond the scope of this study are required

350 to confirm the presence of DMIs.

15

351

352   We examined female longevity postmating with males that were more or less genetically related to them

353   as defined by physical distance. These results from the longevity assay were the inverse of our

354   hatchability assays. Females originating from the central locations (i.e. location 7 and 8) did not seem as

355   affected by heterotypic matings compared to females from the northern and southernmost locations (i.e.

356   locations 2, 3, 12). Previous laboratory evolution studies indicate that females develop 'resistance'

357   against males they coevolve with in the same environment (Arbuthnott et al. 2014). Our findings may

358   support this theory in natural populations, however, due to the low number of lines we tested, our study is

359   possibly lacking the power to appropriately detect the effects of this particular extrinsic postmating barrier.

360

361   We did not find any evidence that egg laying rates or remating rates influenced the reproductive success

362   in a systematic way with regard to these isofemale lines from the southeast United States and Caribbean

363   Islands. However, the lack of evidence from our study does not imply that behaviors are not influential

364   postmating reproductive barriers. Current views of speciation view the process as a sliding continuum

365   where speciation can move forward or step back and may even be arrested at intermediate stages

366   (Seehausen et al. 2014). Depending on the driving force of speciation, different types of reproductive

367   barriers form at particular stages (Seehausen et al. 2014) thus it may be that postmating behaviors could

368   be of importance at other stages in the speciation continuum in which case other species in the

369   *Drosophila* genus may be better candidates to further investigate this question.

370

371   While our findings contribute to the ever growing breadth of knowledge about sexual isolation and

372   speciation, it also sheds light on the complexity of the interplay between different isolating mechanisms

373   and genetic admixture. Overall our data suggests that long-term postmating consequences in terms of

374   offspring fitness and female lifespan reduction are of greater influence in this particular incipient sexual

375   isolation scenario when compared to short-term postmating behavioral responses such as egg laying and

376   remating receptivity. We have also identified the border between the United States and Caribbean islands

377   as a potential region where *D. melanogaster* populations are particularly admixed leading to interesting

378   interactions between partial isolating mechanisms. Greater genetic admixture in flies originating from this

16

379   area could promote the lower hatchability of eggs laid by females from these populations if American and

380   Caribbean flies are genetically distinct enough to increase the possibility of DMIs occurring. The same

381   genetic admixture could also be contributing towards female hardiness against harm from mating with a

382   wider range of genetically diverse males, which in turn can compensate for lower hatchability by

383   increasing reproductive lifespan. Genome resequencing efforts of *D. melanogaster* individuals from this

384   study system will help determine the amount of genetic mixing occurring in the southeast US and

385   Caribbean islands.

386

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484

485   ***Figure Legends***

486   FIGURE 1: Map of locations used in postmating assays

487

488   FIGURE 2: Egg counts of females mated with A) American males and B) Caribbean males. Hatchability

489   of females mated with C) American males and D) Caribbean males. Each box plot is a isofemale line

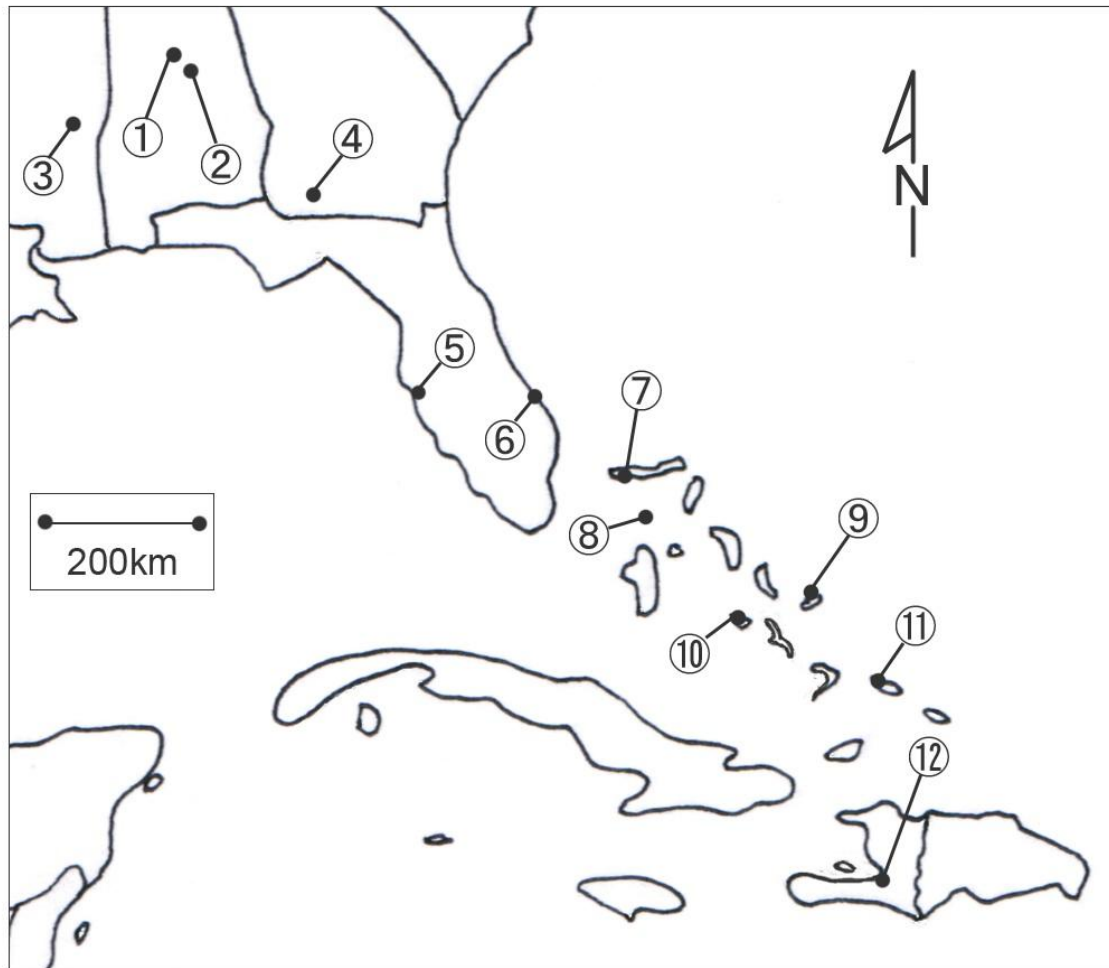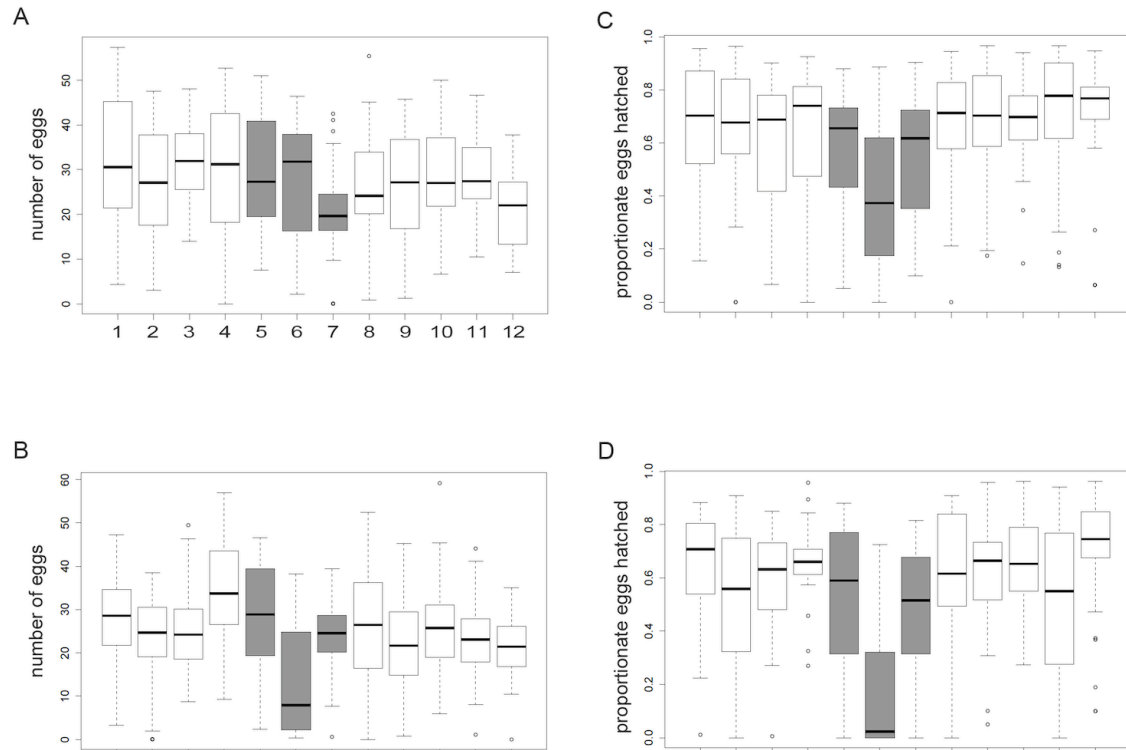490   arranged from the northernmost location (left) to the southernmost location (right)

491

492   FIGURE 3: Survival curves of females from line 2-2 after experiencing homotypic (solid line) or

493   heterotypic (dashed line) matings

494

495   FIGURE 4: Survival curves of females from line 12-2 after experiencing homotypic (solid line) or

496   heterotypic (dashed line) matings

497

498   FIGURE 5: Survival curves of females from line 3-1 after experiencing homotypic (solid line) or

499   heterotypic (dashed line) matings

500

501   FIGURE 6: Survival curves of females from line 7-2 after experiencing homotypic (solid line) or

502   heterotypic (dashed line) matings

503

504   FIGURE 7: Survival curves of females from line 8-1 after experiencing homotypic (solid line) or

505   heterotypic (dashed line) matings

506

507

508   FIGURE1: Map of locations used in postmating assays

FIGURE 2: Egg counts of females mated with A) American males and B) Caribbean males. Hatchability

of females mated with C) American males and D) Caribbean males. Each box plot is a isofemale line

arranged from the northernmost location (left) to the southernmost location (right)
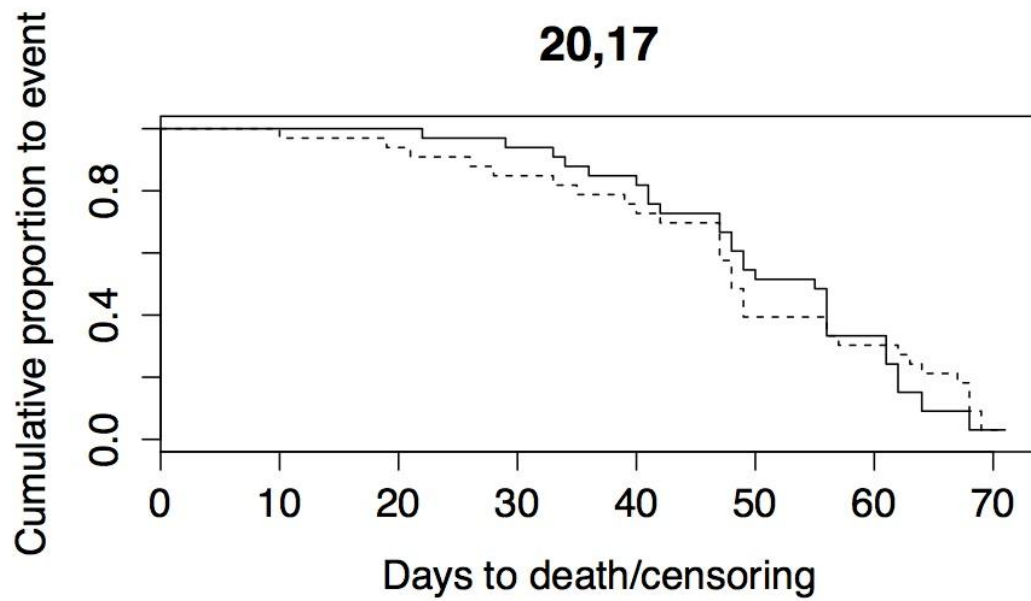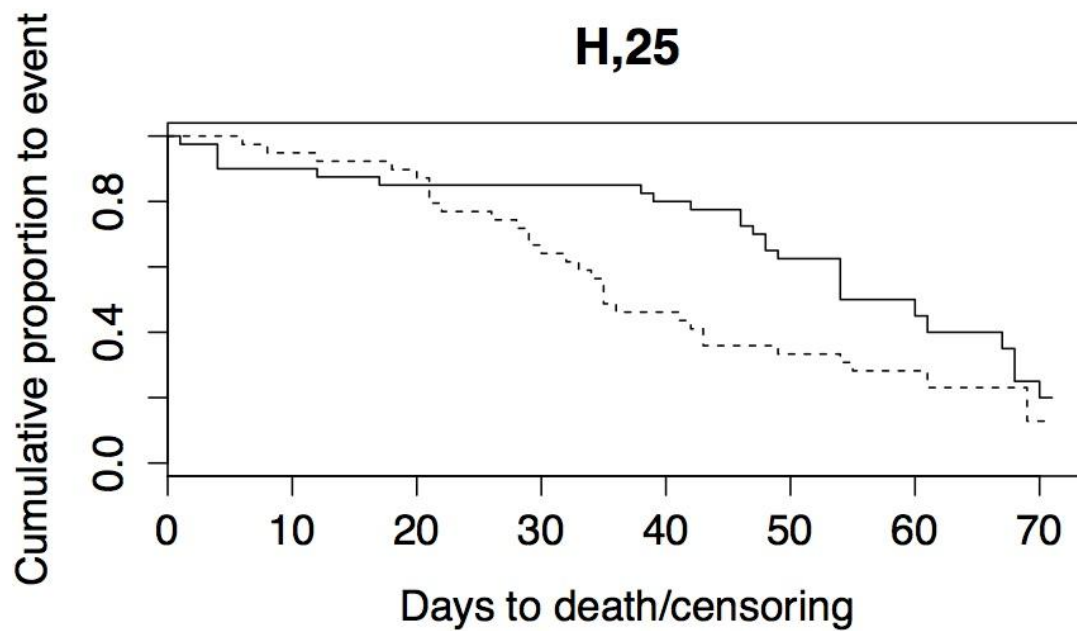
20,17

514

515 FIGURE 3: Survival curves of females from line 20,17 after experiencing homotypic (solid line) or
516 heterotypic (dashed line) matings

517

H,25

518
519 FIGURE 4: Survival curves of females from line H,25 after experiencing homotypic (solid line) or
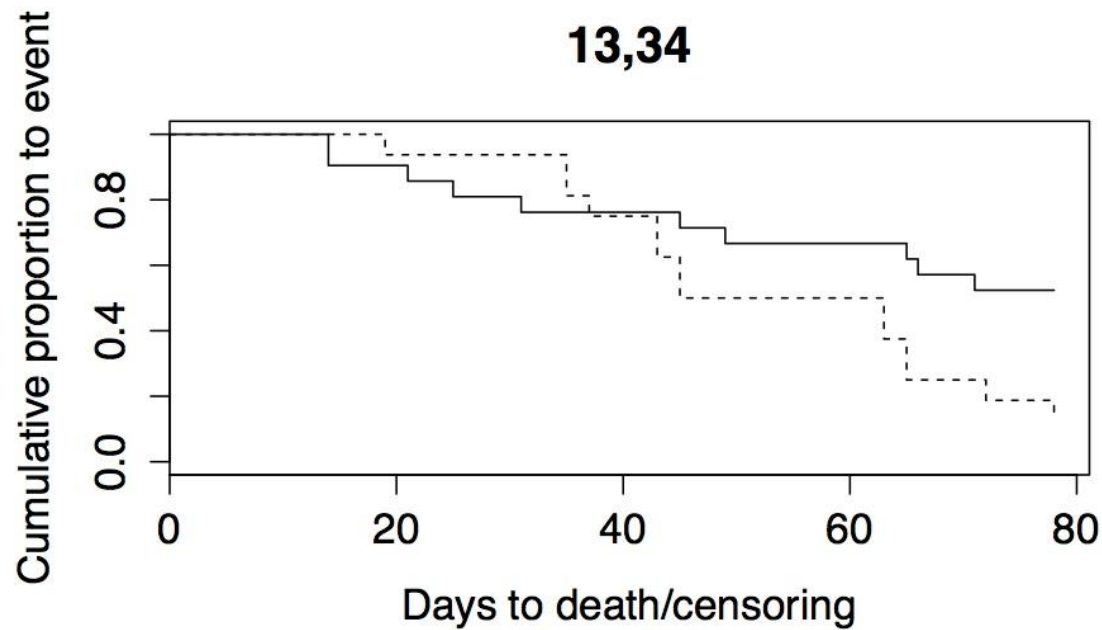520 heterotypic (dashed line) matings

521

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522
523 FIGURE 5: Survival curves of females from line 13,34 after experiencing homotypic (solid line) or
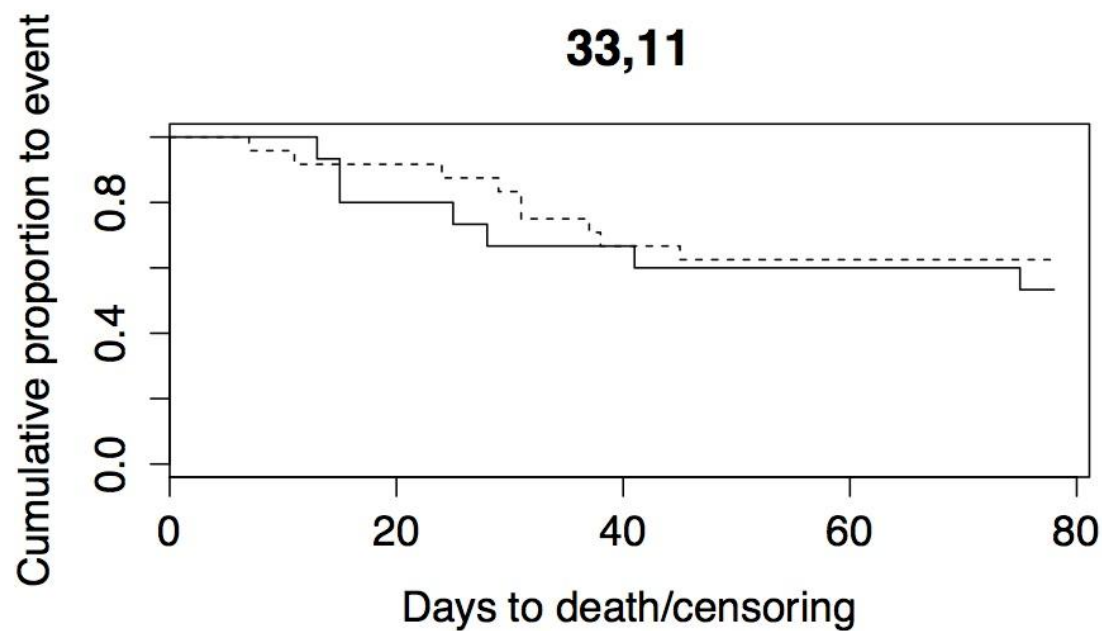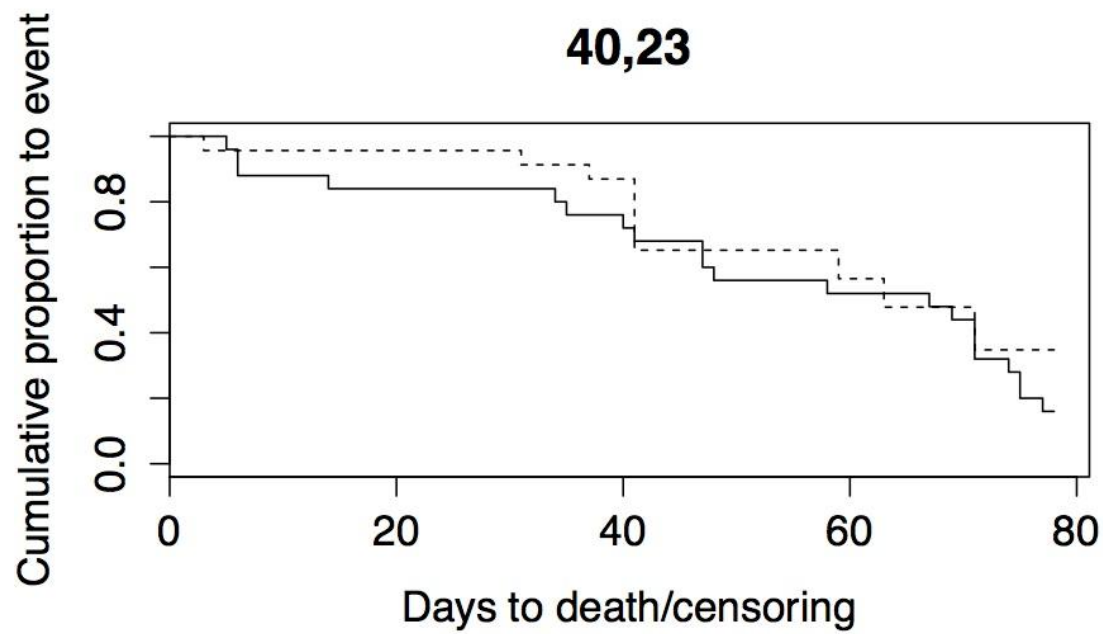524 heterotypic (dashed line) matings

525

526
527 FIGURE 6: Survival curves of females from line 33,11 after experiencing homotypic (solid line) or
528 heterotypic (dashed line) matings

25

FIGURE 7: Survival curves of females from line 40,23 after experiencing homotypic (solid line) or heterotypic (dashed line) matings